FREEZING TOLERANCE AND SURVIVAL EXPERIMENTS WITH VARIOUS INTERTIDAL ORGANISMS FROM KACHEMAK BAY, ALASKA

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A

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ABSTRACT

Intertidal organisms at high latitudes experience multiple stresses created by freezing, including ischemia, free water reduction, and distortion and destruction of cells, and in response have adapted behavioral and physiological solutions. This study examined the response of intertidal organisms in Kachemak Bay, Alaska to freezing through laboratory experiments and field studies. *Mytilus trossulus*, *Balanus glandula*, *Protothaca staminea* and various limpets (Lottidae) survived freezing conditions to –10 and –20°C, depending on the season. *Mytilus trossulus* and *B. glandula* survived multiple freeze events at –10°C. Seasonal freeze response was not induced by exposure to low air temperature in *M. trossulus*. Exposure to 0°C was not fatal to any of the species studied: *M. trossulus*, *B. glandula*, *P. staminea*, limpets, *Fusitriton oregonensis*, *Katharina tunicata* and *Leptasterias hexactis*. Preliminary results suggest that *M. trossulus* and *P. staminea* have an ice nucleator. Freezing avoidance may be one cause for the differences seen in seasonal distribution patterns of *F. oregonensis*, *Nucella lima*, *Onchidella borealis*, *Siphonaria thersites* and *Littorina sitkana*. The current study demonstrated that intertidal organisms in this region exhibit differing responses to freezing. Some organisms survive freezing conditions by freeze tolerance, while others may avoid it by moving lower in the intertidal.

Keywords: Alaska, intertidal, freezing, mussel, *Mytilus*, clam, barnacle, *Protothaca*, *Balanus*, supercooling

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INTRODUCTION

The intertidal zone is a challenging habitat for marine organisms because of the range of physical conditions encountered on a daily, lunar-cycle, seasonal, and annual basis (7). As the tide flows in and out, there are changes in temperature, salinity, and availability of oxygen (46). In arctic and subarctic regions, subzero temperatures may cause organisms to freeze during exposure at low tide and then quickly thaw when the tide returns (46). In Alaska, this freezing cycle can occur twice a day, for roughly two weeks each month for 5-7 months.

Freezing creates multiple stresses on intertidal invertebrates. Freezing decreases the water in which the solutes (ions) are dissolved, which increases the concentrations of ions inside the cell, thus setting up an osmotic gradient stress. Ischemia stress occurs when freezing stops transport of oxygen within cells (46). There is also mechanical stress, which occurs when intracellular ice forms, causing cell death or cell distortion (37). These stresses can cause damage and death to organisms, unless the organisms can adapt physiologically or behaviorally to freezing temperatures (31, 38).

One behavioral strategy for mobile invertebrates is to migrate to the upper subtidal before the tide recedes and the temperature drops. The intertidal snail, *Nassarius obsoletus*, migrates subtidally because it cannot survive temperatures below –7°C (41). Another behavioral strategy might be to change distributions between higher and lower intertidal zones during summer and winter months; *N. obsoletus* actively migrates subtidally and below the mud surface during the winter and then back to the surface in the summer (37).

Freeze tolerance, freeze avoidance and ice control are physiological adaptations. Certain fish and terrestrial organisms have glycoproteins and protein antifreeze molecules to assist in freeze tolerance, avoidance and control. Fish antifreezes work in a non-colligative manner, lowering the freezing point of blood without changing the melting point (21). Many insects survive freezing by preventing the freezing of their body solutions; these insects supercool, thus remaining at a

metastable state at temperatures at or below its freezing point (15). For organisms living in the rocky intertidal zone a daily temperature change of 10-20°C is not uncommon, while most organisms from other habitats have a narrow range of ~10°C within which their biochemical systems function and a greater difference in temperature can lead to death (7). Freeze tolerance in many intertidal organisms is defined by an ability to be frozen without significant tissue damage. The barnacle, *Balanus balanoides,* can survive with up to 80% of its body fluids frozen and the mussel, *Mytilus edulis,* with up to 62-64% frozen (16, 31, 51). By forming ice in the extracellular spaces, there is less damage to the organism than if ice formation occurred intracellularly (37). Molluscs have been shown to have a hemolymph ice nucleator protein, which induces ice formation at relatively high subzero temperatures in extracellular spaces. Shelled invertebrates that close completely can prevent ice propagation from the external environment (37). In addition, shells may impede heat transfer, which would give ice-crystals time to grow (32). Ice crystals can be viewed inside the shell and muscle tissue of intertidal invertebrates when their shells are opened during winter low tides (32, and pers. obs).

Studies also have shown that some organisms exhibit a seasonal physiological adjustment to freeze tolerance (2, 13, 16, 35), with lethal temperatures varying by season (37). There also are indications that geographic distribution and zonation influence freeze tolerance, with higher latitude and higher intertidal organisms having greater cold tolerances (45).

Non-intertidal organisms such as insects (4, 6, 15, 24, 25, 33) and fish (21, 26, 43) can have cryoprotection and antifreeze compounds that allow them to survive freezing. Thus far studies have identified some ice nucleators in intertidal organisms (37), but have not shown that intertidal organisms have sufficient concentrations of any compounds with cyroprotectant/antifreeze properties that would allow them to endure subzero-freezing temperatures exhibited in some terrestrial and submerged polar organisms (12, 13, 37, 46). The mystery of freeze tolerance mechanisms in the intertidal zone still requires investigation.

Little work has been done on the affects of freezing air temperatures on algae and animals in the Alaskan intertidal. Most investigations of freeze tolerance or

response in intertidal zones have been done in the northeast United States (31, 32, 37, 39, 51) and in Europe (1, 14, 16, 17, 38, 44, 45, 48, 49). Many Alaska coastal areas often experience subzero temperatures during the winter while having extreme tidal cycles (up to ±9m range). Therefore, the ability to cope with freezing temperatures is clearly an important component of living in such intertidal environments. Because of the extreme temperatures and low tides, Alaska is an ideal setting to examine freeze tolerance in intertidal organisms.

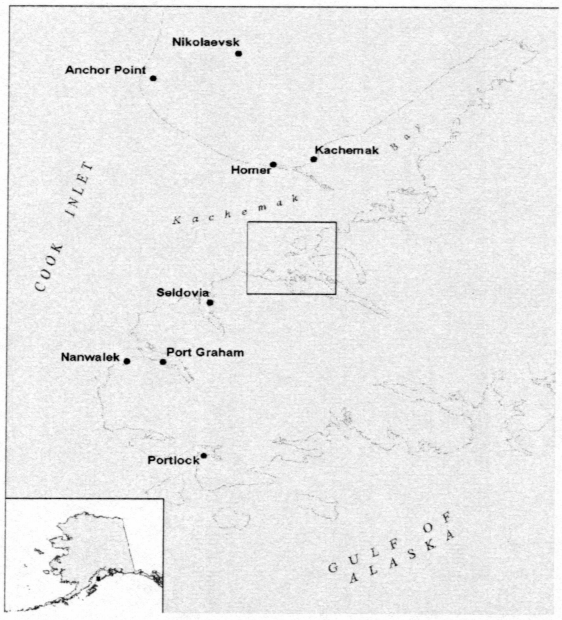
The over-arching goal of this study was to investigate the effects of freezing from exposure to low air temperature on several intertidal invertebrates from the rocky intertidal of Kachemak Bay, Alaska. The objectives of this study were to: (1) Determine if the barnacle, *Balanus glandula*, the clam, *Protothaca staminea*, and the mussel, *Mytilus trossulus* survive freezing temperatures (0, -10 and -20°C) equally; (2) Determine if *B. glandula*, *P. staminea*, and *M. trossulus* have a seasonal aspect to their freezing survival; (3) Determine if *M. trossulus* and *B. glandula* can survive repeated freezing at -10°C during the winter; (4) Determine if selected organisms found in the upper intertidal (*M. trossulus*, *B. glandula*, and Lottidae) survive better than those found lower in the intertidal (*P. staminea*, *Katharina tunicata*, *Leptasterias hexactis*, and *Fusitriton oregonensis*); (5) Determine if survival of *M. trossulus* differs by habitat (intertidal, high dock, and dock submerged) at freezing temperatures (0, -10 and -20°C); (6) Determine if the average supercooling point (the lowest point of liquid state below the freezing point) differs between *M. trossulus*, *P. staminea*, and seawater; (7) Determine if the average supercooling point of *M. trossulus* differs by habitat (intertidal, high dock, and dock submerged); and (8) Determine if type, density and mobility of species found in the upper and lower intertidal differ between seasons.

MATERIALS AND METHODS

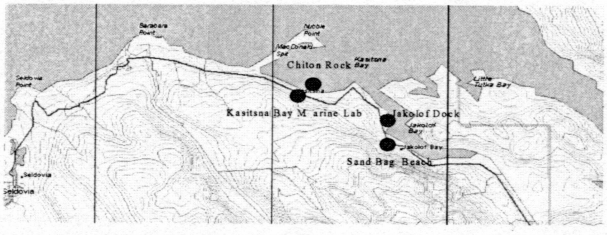
Study site

Kachemak Bay, Alaska (N 59° 8.0' - Figure 1) presents an ideal location for study of cold tolerance in intertidal organisms due to the low air temperatures and large tidal fluctuations. Samples for all laboratory experiments were collected from

the rocky intertidal from either near the Kasitsna Bay Laboratory or from a site near Sand Bag Beach in Jakolof Bay. Organisms were collected from +3 m to 0 m tidal height (except for *Fusitriton*, which had to be collected subtidally because they had not yet migrated into the intertidal or were in very low densities).



A.



B.

Figure 1; Study area: (A) Location of Kachemak Bay in southcentral Alaska. (B) Study locations within Kachemak Bay are Kasitsna Bay, Jakolof Dock, and Sand Bag Beach.

Observations during August 2001 estimated that study organisms were exposed to air for an average of 3.5 to 6 hours per tidal cycle. Tides in this area have a mean range of 4.5 m and an extreme range of 9-10 m (10, 11). Southcentral Alaska has mixed semidiurnal tides. In Kachemak Bay, as reported for Seldovia, there are recorded low temperature extremes of –31°C (weather.com).

Freeze tolerance

Several intertidal species were chosen to test for freeze tolerance during different times of the year. The mussel, *Mytilus trossulus*, and barnacle, *Balanus glandula* were chosen because of previous studies on similar species (13, 14, 16, 17, 31, 51) and their numerical dominance in the mid-upper regions of the intertidal zone in Kachemak Bay. The clam *Protothaca staminea* was examined at the request of the Alaska Department of Fish and Game and as a comparison bivalve for *M. trossulus*. The chiton *Katharina tunicata*, sea star *Leptasterias hexactis* and Lottidae (hereafter referred to as limpets) were chosen due to their dominant presence in the intertidal and their mobility. The triton snail *Fusitriton oregonensis* was chosen because it exhibits a seasonal distribution, with higher densities in the intertidal in the summer than during the winter.

Studies were conducted from August 2001 to May 2003. Depending on collection month, either 10 (August 2001, December 2001, May 2002, August 2002) or 20 (December 2002, January 2003, March 2003, May 2003) individuals of select species were collected from the field and transported within four hours to the Kasitsna Bay Laboratory. To reduce stress created after collection, organisms were kept in running ambient seawater tanks for at least 24 hours prior to each experiment. Each organism was used only once, except for the repeated freezing experiments on *B. glandula* and *M. trossulus*. For years 2001 to 2003, *M. trossulus*, *B. glandula* and *P. staminea* were collected and tested. In year 2003, *K. tunicata*, *L. hexactis*, *F. oregonensis* and various species of limpets were added. Identification of limpets to species was difficult, so they were grouped together as limpets but included *Lottia pelta*, *Tectura scutum*, and *Tectura persona*.

Survival experiments were conducted at 0, -10 and -20°C temperature conditions. A pilot study determined that there was no survival at -30 or -40°C, so experimentation was to limited to temperatures equal to or greater than -20°C. To assess survival, 10 or 20 individuals were placed in plastic containers (0.23 – 8.64 m^3) in either refrigerators or freezers set to 0°C, -10°C, or -20°C. Organisms were removed from the seawater tanks and gently shaken to remove excess water to mimic

natural conditions. *Mytilus trossulus*, *Balanus glandula* and limpets were held in the freezer/refrigerator for 6 hours and *Leptasterias hexactis*, *Fusitriton oregonensis*, *Protothaca staminea* and *Katharina tunicata* for 3.5 hours. These times were selected to represent the length of average air exposure under natural conditions. *Mytilus trossulus*, *B. glandula, K. tunicata, F. oregeonensis, L. hexactis* and limpets were placed in dry containers to mimic natural conditions, while *P. staminea* was placed in a container filled with damp sand to mimic natural conditions. The insulation of sand usually kept *P. staminea* at least 5°C higher than the other species. Visual observations of ice demonstrated that water froze at the lower two temperature treatments. After freezing, the animals were placed in a running seawater tank for 6 or 8.5 hours (to complete a full tidal cycle) for recovery. Once the recovery period was complete, survival was assessed. Southward (45) and Churchill and Storey (12) assessed survival in molluscs by probing the foot or other appropriate muscle while looking for a reaction. Williams (51) found that *Mytilus edulis* gaped open when it was dead because the adductor muscles were unable to contract. In the current study, death was recorded when *B. glandula*, *M. trossulus* and *P. staminea* gaped, *K. tunicata* failed to curl into a ball when placed on its back, *L. hexactis* would not move its tube feet and was limp, and *F. oregonensis* and limpets would not move their foot in an effort to right themselves when placed on their backs. The number of individuals alive, as assessed by the survival test, was used as an index of a species ability to survive one tidal cycle at the experimental temperature.

To assess long-term freeze tolerance, select individuals were subjected to multiple freeze events. *Mytilus trossulus* and *Balanus glandula* were chosen for repeated-freezing tests due to their length of exposure and dominance in the upper intertidal. For the repeated-freezing experiment, conducted in January 2003, an individual *M. trossulus* or *B. glandula* that survived an initial freezing to -10°C was then subjected to repeated freezing events. Twenty individuals were placed in the -10°C freezer for six hours, then back in ambient, running seawater for six hours. The freezing and recovery sequence was repeated four times. Survival was assessed as stated above.

To assess if habitat had any conditioning affect on the survival of *Mytilus trossulus*, twenty individuals were collected from three habitats with different aerial exposures. *Mytilus trossulus* was chosen because it occurs in a variety of locations with different aerial exposures. Twenty individuals were collected from the underside of the floating dock at Jakolof Bay. This dock moves up and down with the tide, with lower portions never exposing attached mussels to air. Twenty individuals were collected from the high dock, a section of the floating dock walkway that leads to the beach. The upper location is exposed to air at some low tides (+2.4m). Twenty intertidal individuals were collected from Sand Bag Beach at a +3.0m tidal height. The habitat experiment, conducted in January 2003, was performed using the organism survival protocols described above.

Supercooling point

To assess supercooling points of various intertidal organisms, individuals were collected from the intertidal and adjacent sand flat at Sand Bag Beach and stored in running seawater tanks similar to the organism survival experiments. A small hole (~2mm) was hand drilled using the tip of a drill bit near the umbo in each *Mytilus trossulus* and *Protothaca staminea*, similar to Murphy & Pierce (39). A pilot study demonstrated that a small hole drilled into a bivalve umbo did not effect survival over the short term (up to 1 week). HOBO H8 (Onset) dataloggers were used to determine the supercooling point temperatures of individuals. The internal thermistor of the datalogger was extended out of each logger housing and inserted into the umbo hole of an individual. The supercooling point of seawater was also run as a control. For this, seawater was poured into ice cube trays with a thermistor placed in each section to monitor temperature. Individual invertebrates were placed into an insulated container (to reduce the oscillation in temperature that a freezer naturally creates) and then into a -20°C freezer. Ten replicate individuals were done per trial. An additional data logger recorded ambient temperature in the freezer. The cooling rate was similar to the rate of natural conditions as determined by a pilot study; cooling rates were up to 20°C/hour in nature while experimental rates averaged 15°C/hour (Patterson unpublished data). During each trial, individuals were subjected to -20°C for

approximately three hours, and then thawed at room temperature for approximately 2 hours. Survival was assessed as described above. Only those individuals alive at the end of each trial were used for supercooling point calculations. A supercooling point is defined as the coldest point reached before the release of heat due to the latent heat of fusion, which is shown as a spike on a temperature graph.

To assess if there was any thermal hysteresis present in the hemolymph of *Mytilus trossulus*, five individuals were collected from the intertidal near Sand Bay Beach and stored in running seawater tanks. A small hole was hand drilled, as described above, and a syringe was placed into the organism withdrawing hemolymph. The hemolymph was stored in tubes in a -20°C freezer until analysis (at the University of Alaska, Fairbanks) for thermal hysteresis between freezing and melting points, indicating molecular activity preventing growth of ice. Thermal hysteresis, diagnostic of the presence of antifreeze activity, was determined using the technique of DeVries (23). The solution to be tested is seeded with an ice crystal, and then the temperature is increased until only a very small ice crystal is visible under the microscope. This temperature reflects the melting point and equilibrium freezing point of the sample. If the temperature is then lowered a small amount (0.01 - 0.02°C), the crystal will begin to grow if antifreeze proteins are not present. If antifreeze proteins are present the crystal will not grow until the temperature has been lowered to the hysteretic freezing point (i.e. melting point ≠ freezing point). Because a seed crystal is present in the sample as the temperature is lowered, the temperature where the crystal grows (hysteretic freezing point) is not the nucleation temperature (supercooling point) of the sample.

Distribution of intertidal organisms

To determine if the distribution and density of intertidal organisms varied seasonally, six quadrats (0.3x0.3 m) were haphazardly placed in each of the upper and lower intertidal zones during the months of January, March, and May 2003. The surveys were conducted at low tide along 8 m of beach near Chiton Rock (Figure 1). Barnacles characterized the high zone (1.2 m to -0.6 m tidal height), while mussels and rockweed characterized the low zone (-0.7 m to –1.2 m tidal height). All major

species that could be visually observed within the quadrat were quantified and identified to species.

Statistical analysis

SAS (Version 8.0) was used for all statistical analysis. Organism survival was calculated as percent alive, but due to a pseudoreplicated design, statistics could not be performed on the percent alive data. As there was no pseudoreplication for *Mytilus trossulus* by habitat (dock, high dock, intertidal), an ANOVA followed by Tukey's post-hoc pairwise comparison was run on the percent alive data. Only those organisms that survived the supercooling point experiment were used to calculate the average supercooling point. An ANOVA was then run on these data to determine if there was a difference by species, location, and time of year, followed by Tukey's post-hoc pairwise comparison. An ANOVA, followed by Tukey's post-hoc pairwise comparison, was also run on the distribution quadrat data, with the variables zone, species (genus) and mobility.

RESULTS

Freeze tolerance

Organisms were frozen at the two lower temperatures. Percent survival of *Mytilus trossulus*, *Balanus glandula* and *Protothaca staminea* varied by exposure, temperature and season of collection (Table 1). At -20°C, the lowest temperature condition tested, *P. staminea* had greater survival than *M. trossulus* and *B. glandula*, with a mean of 90% survival in December 2001, 70% in May 2002, 30% in December 2002, and 65% in May 2003. This compares to a mean survival for *B. glandula* of 40% in December 2001 and 83% in December 2002 and for *M. trossulus* 30% in December 2001 and 63% in December 2002. Overall, *P. staminea* survived to –20°C conditions in May and December, while *M. trossulus* and *B. glandula* survived to –20°C only in December.

Seasonal variation was also seen at -10°C for all organisms tested. In all trials except for August 2002, some *Protothaca staminea* individuals survived at -10°C conditions, averaging 60% ±37% survival (Table 1B). *Mytilus trossulus* and *Balanus glandula* had more mixed results with greater survival from December 2002 to May 2003 than from August 2001 to December 2002. From December 2002 to May 2003,

Table 1: Freezing survival of *M. trossulus*, *B. glandula* & *P. staminea*. (A) Percent survival following cooling to 0, -10 and –20°C of *Protothaca staminea*, *Balanus glandula* and *Mytilus trossulus* August 2001-Apr/May 2003. Air temperatures are from the Homer Airport (located beside Kachemak Bay). (B) Percent seasonal survival means (±1s.d.) of *P. staminea*, *B. glandula* and *M. trossulus*.

		Protothaca		*Balanus*		*Mytilus*		Temperature (Homer Airport)				
	(°C)	*staminea*	n	*glandula*	n	*trossulus*	n	Mean High °C	Mean Low °C	Mean °C	High °C	Low °C
Aug-01	-20	0.0%	9	0.0%	10	0.0%	9					
(summer)	-10	22.5%	9	10.0%	10	10.0%	9	13	5.6	9.3	16.7	0
	0	100.0%	10	100.0%	10	100.0%	10					
Dec-01	-20	90.0%	10	40.0%	10	30.0%	10					
(winter)	-10	80.0%	10	56.5%	10	10.0%	10	-3.3	-10.6	-6.9	8.9	-20.5
	0	100.0%	10	100.0%	10	95.0%	10					
May-02	-20	70.0%	10	0.0%	10	0.0%	10					
(summer)	-10	40.0%	10	0.0%	10	0.0%	10	12.9	2.2	7.1	20.6	-3.3
	0	100.0%	10	100.0%	10	100.0%	10					
Aug-02	-20	0.0%	10	0.0%	10	0.0%	10					
(summer)	-10	0.0%	10	10.0%	10	0.0%	10	13.2	6.2	9.7	19.4	-3.8
	0	100.0%	10	100.0%	10	100.0%	10					
Dec-02	-20	30.0%	20	83.3%	30	63.3%	30					
(winter)	-10	85.0%	20	83.3%	30	96.7%	30	1.3	-4.7	-1.7	10.6	-16.7
	0	100.0%	20	100.0%	30	100.0%	30					
Jan-03	-20	0.0%	20	0.0%	20	0.0%	20					
(winter)	-10	50.0%	20	100.0%	20	100.0%	20	3.6	-3.2	0.2	8.9	-16.7
	0	100.0%	20	100.0%	20	100.0%	13					
Mar-03	-20	0.0%	20	0.0%	20	0.0%	20					
(winter)	-10	100.0%	20	100.0%	20	95.0%	20	17.1	-5.8	-2.1	9.4	-16.1
	0	100.0%	20	100.0%	20	100.0%	20					
Apr/May-03	-20	65.0%	20	0.0%	20	0.0%	20					
(summer)	-10	100.0%	20	100.0%	20	100.0%	20	8.1	-0.4	3.8	17.2	-8.3
	0	100.0%	20	100.0%	20	100.0%	20					

A.

		Protothaca			*Balanus*			*Mytilus*		
	(°C)	mean	s.d.	n	mean	s.d.	n	mean	s.d.	n
Summer	-20	33.8%	39.0%	49	0.0%	0.0%	50	0.0%	0.0%	49
mean	-10	40.6%	42.8%	49	30.0%	46.9%	50	27.5%	46.9%	49
	0	100.0%	0.0%	50	100.0%	0.0%	50	100.0%	0.0%	50
Winter	-20	30.0%	42.0%	70	30.8%	39.7%	80	23.3%	30.2%	80
mean	-10	78.8%	21.0%	70	85.0%	20.5%	80	75.4%	43.7%	80
	0	100.0%	0.0%	70	100.0%	0.0%	80	98.8%	2.5%	73
Overall	-20	31.9%	37.8%	119	15.4%	30.8%	130	11.7%	23.4%	129
mean	-10	59.7%	37.3%	119	57.5%	44.6%	130	51.5%	49.8%	129
	0	100.0%	0.0%	120	100.0%	0.0%	130	99.4%	1.8%	123

B.

M. trossulus and *B. glandula* had a mean survival greater than 80% at -10°C. While from December 2001 to August 2002, the highest mean survival at -10°C was 56% for *B. glandula* and only 10% for *M. trossulus*. In 2001 and 2002 *B. glandula* and *M.*

trossulus tended to have better survival to -10°C in the winter (December, January, March) months than in the summer (May and August) months (Table 1B).

At the highest temperature tested, 0°C, all individuals survived freezing with only one exception. In December 2001, one *Mytilus trossulus* individual died (Table 1). There was no seasonal difference in survival for any species at 0°C.

Repeated freezing of *Mytilus trossulus* and *Balanus glandula* showed that these species are resilient to multiple freezing events (Table 2). With repeated freezing at -10°C, *M. trossulus* and *B. glandula* had 100% survival if the individual survived the first freezing event. Both species were able to survive at least four sequential freezing events (Table 2).

Table 2: Survival after repeated freezing/thawing cycles of *Mytilus trossulus* (n=20) and *Balanus glandula* (n=20) at –10°C in January 2003. Specimens were frozen for six hours and then allowed to recover in ambient seawater for six hours for a total of four cycles.

	x1 freeze	x2 freeze	x3 freeze	x4 freeze
Mytilus trossulus	95.00%	100.00%	100.00%	100.00%
Balanus glandula	100.00%	100.00%	100.00%	100.00%

Survival of *Fusitriton oregonensis*, *Katharina tunicata*, *Leptasterias hexactis*, limpets (*Lottia pelta*, *Tectura scutum* and *Tectura persona*), *Mytilus trossulus*, *Balanus glandula* and *Protothaca staminea* varied temporally and by exposure temperature (Table 3). Only *P. staminea* survived the lowest temperature, -20°C, with a mean survival of 65% in April/May 2003. The greatest survival for all species at -10°C occurred in April/May 2003, which further supports the hypothesis that there is a seasonal component to freeze tolerance. The overall mean survival (at -10°C) of all organisms was 79% ±28% in April/May, while it was only a 51% ± 49% in January 2003 and 64%±47% in March 2003. Limpet survival mirrored that of *M. trossulus* and *B. glandula* for season and temperature (Table 3).

Table 3: Percent survival of *Fusitriton oregonensis*, *Katharina tunicata*, *Leptasterias hexactis*, limpets, *Mytilus trossulus*, *Balanus glandula* and *Protothaca staminea* in January, March and April/May 2003. A period mark means that no data were collected for that species at that time. Animals were collected from Kasitsna Bay & Sand Bag Beach.

	(°C)	*Fusitriton oregonensis*	n	*Katharina tunicata*	n	*Leptasterias hexactis*	n	Limpet	n	*Mytilus trossulus*	n	*Balanus glandula*		*Protothaca staminea*	n	Overall average	Overall s.d
Jan-03	-20	0.0%	20	0.0%	20	0.0%	20	0.0%	25	0.0%	20	0.0%	20	0.0%	20	0.0%	0.0%
	-10	5.0%	20	0.0%	20	0.0%	20	100.0%	16	100.0%	20	100.0%	20	50.0%	20	50.7%	49.2%
	0	100.0%	20	100.0%	20	100.0%	20	100.0%	15	100.0%	13	100.0%	20	100.0%	20	100.0%	0.0%
Mar-03	-20	.	.	.	.	.	.	0.0%	10	0.0%	20	0.0%	20	0.0%	20	0.0%	0.0%
	-10	0.0%	20	50.0%	20	0.0%	20	100.0%	15	95.0%	20	100.0%	20	100.0%	20	63.6%	47.0%
	0	100.0%	20	100.0%	20	100.0%	20	100.0%	20	100.0%	20	100.0%	20	100.0%	20	100.0%	0.0%
Apr/May-03	-20	.	.	.	.	.	.	0.0%	20	0.0%	20	0.0%	20	65.0%	20	16.3%	32.5%
	-10	33.0%	15	57.0%	20	60.0%	20	100.0%	20	100.0%	20	100.0%	20	100.0%	20	78.6%	28.1%
	0	100.0%	20	100.0%	20	100.0%	20	100.0%	20	100.0%	20	100.0%	20	100.0%	20	100.0%	0.0%

Habitat had no effect on the freezing survival of *Mytilus trossulus* (ANOVA, F=0.64, df=2, p=0.5351, Table 4). All individuals tested, regardless of habitat, survived similarly at the various temperatures (Table 4). However, there was a significant difference in *M. trossulus* survival by temperature (ANOVA, F=482.65, df=2, p=<0.0001). Significantly more *M. trossulus* survived at 0 and -10°C than -20°C (Tukey, alpha-0.05, Table 4).

Table 4: Survival of *Mytilus trossulus* by habitat (n=20). Intertidal individuals were collected from Sand Bag Beach, while High Dock and Dock (in water) individuals were collected from Jakolof dock. Tidal height was +3, +2,4, and 0 m for intertidal, high dock and dock (in water) habitats respectively.

	***Mytilus* intertidal**		***Mytilus* High Dock**		***Mytilus* Dock in Water**		**All *Mytilus* combined**	
°C	mean	s.d.	mean	s.d	mean	s.d	mean	s.d
-20	0.0%	0.0%	15.0%	19.2%	0.0%	0.0%	5.0%	9.0%
-10	100.0%	0.0%	100.0%	0.0%	100.0%	0.0%	100.0%	0.0%
0	100.0%	0.0%	95.0%	10.0%	100.0%	0.0%	98.3%	3.0%

Supercooling Point

The average supercooling point of *Mytilus trossulus* did not significantly vary on a seasonal scale (Table 5). Average supercooling point varied from –3.41 ±1.81°C (December 2002) to –3.33 ± 1.15°C (April/May 2003). The supercooling point of *Protothaca staminea* also did not vary significantly but did have a slightly lower average supercooling point in March 2003 (-4.09 ± 1.78°C) compared to January 2003 (-2.98 ± 1.94°C) and Apr/May 2003 (-2.79 ± 0.55°C) (Table 5; ANOVA, F=0.82 df=3 p=0.4886). There was a significant difference in average supercooling point by species and the seawater control (*M. trossulus*, *P. staminea* and seawater - ANOVA, F=33.55 df=3 p=<0.0001). Across all months sampled *M. trossulus* averaged –3.48 ± 0.30°C, while *P. staminea* averaged –3.29 ± 0.70°C. There was also a significant difference in average supercooling points between the two species tested and seawater (Table 5; Tukey, p<0.05), with seawater having a lower average supercooling point of –7.68 ± 1.57 °C.

Table 5: Average supercooling point of *Mytilus trossulus*, *Protothaca staminea* and seawater for December 2002 to Apr/May 2003. Period marks mean that no data were collected for that month. Individuals were collected from Sand Bag Beach. Hobo H8 Dataloggers were used to record supercooling points.

	Mytilus trossulus			***Protothaca staminea***			**Seawater**		
	mean (C)	s.d	n	mean (C)	s.d	n	mean (C)	s.d	n
Dec-02	-3.41	1.81	9	.	.	.	.	.	.
Jan-03	-3.28	0.85	5	-2.98	1.94	19	-7.68	1.57	8
Mar-03	-3.93	0.94	9	-4.09	1.78	3	.	.	.
AprMay-03	-3.33	1.15	8	-2.79	0.55	9	.	.	.
Overall mean	-3.48	0.30	31	-3.29	0.70	31	.	.	.

As discussed, the average supercooling point for *Mytilus trossulus* did not significantly vary depending on habitat. However, *M. trossulus* collected from the intertidal did have an average supercooling point of -3.28 ± 0.85°C, which was slightly lower, though not statistically significant, than *M. trossulus* collected from the high dock (-2.44 ±0.80°C, n=11) or *M. trossulus* (-2.85 ±0.64°C, n=9) collected from under the dock submerged (ANOVA, F=2.88, df=2, p=0.0748).

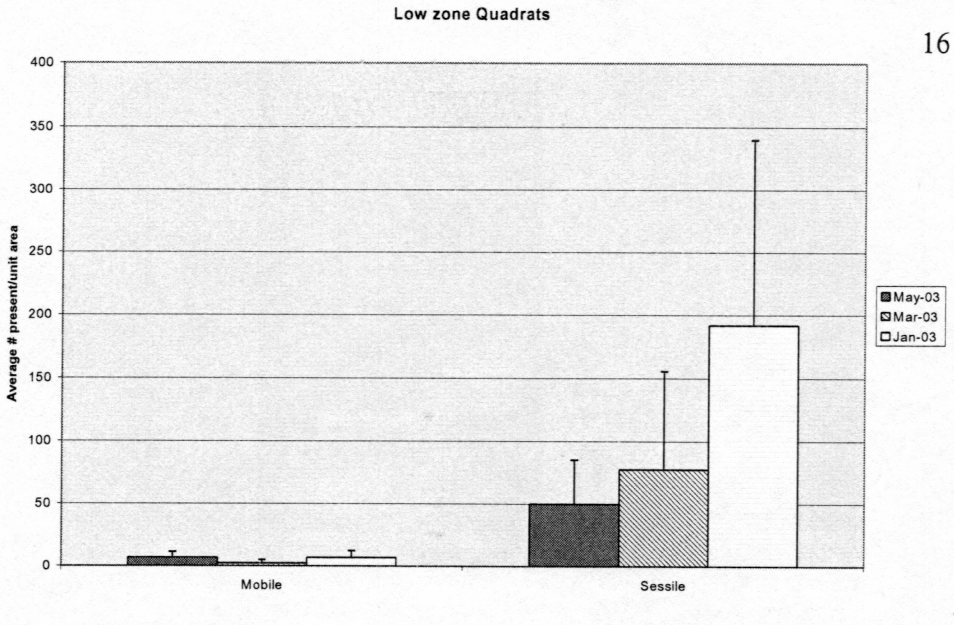
The average thermal hysteresis for *Mytilus trossulus* was –0.248±0.234°C. There was maximum difference between the melting point and freezing point of 0.51 °C, and a minimum difference of 0.18 °C.

Distribution of intertidal organisms

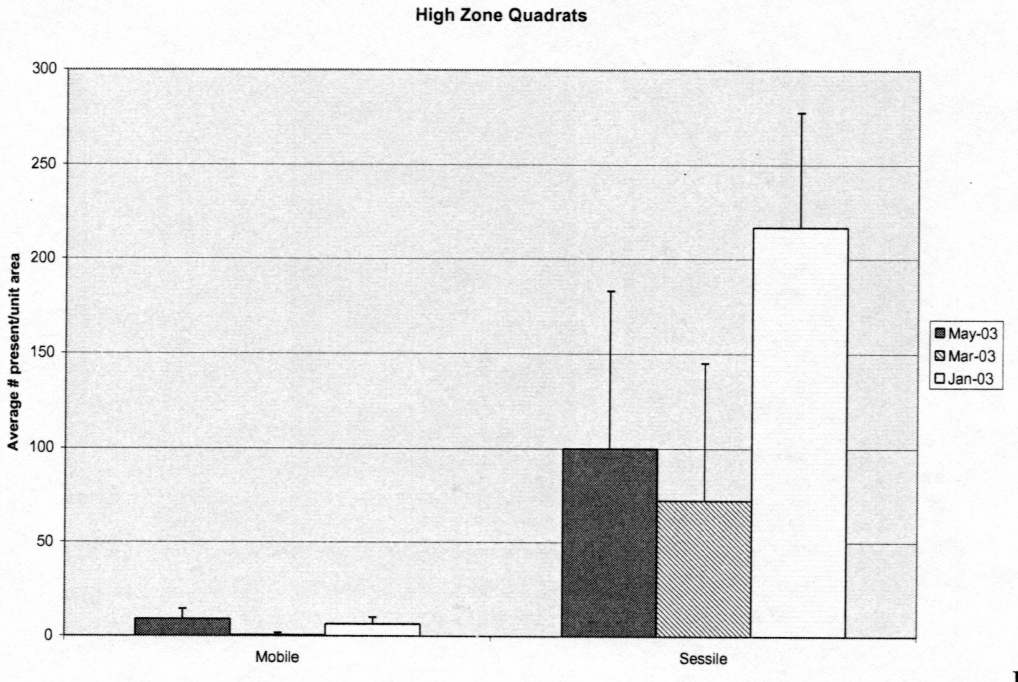
Species, density and mobility of organisms present varied spatially and temporally (Table 6). There was a significant difference in the average density by species present overall (ANOVA, F=18.37, df=16, p=<0.0001), but there was no significant difference between individual species (Tukey α=0.05). A significant seasonal difference also was found in density (ANOVA, F=6.60, df=2, p=0.0022). This significant seasonal difference was between January 2003 and March 2003, and between January 2003 and Apr/May 2003 (Tukey α=0.05), with January having the lowest density. No significant difference was found in average densities of species by zone (ANOVA, F=0.57, df=1, p=0.4518), although it appears that there is a trend for higher abundances of barnacles in the high than in the low zone. *Fusitriton oregonensis*, *Nucella lima* (gastropod), hermit crabs, *Metridium senile* (anemone), *Evasterias troschellii* (sea star), *Mopalia ciliata* (chiton) and the *Urticina crassicornis* (anemone) were only found in the low zone. *Onchidella borealis* (opisthobranch) and *Siphonaria thersites* (false limpet) were only found in the high zone during Apr/May 2003 (Table 6). There was a difference in average density by the mobility of the organisms (ANOVA, F=64.05, df=1, p=<0.0001, Figure 2), with higher densities of sessile organisms.

Table 6: Mean density of organisms in the high and low zone from January 2003 to Apr/May 2003. M=mobile species, S=sessile species. Quadrats (0.30-m x 0.30-m) were haphazardly placed in each the high and the low zones in Kasitsna Bay, near Chiton Rock (n=6/zone).

	Jan-03		Mar-03		Apr/May 03	
Low						
	mean	s.d.	mean	s.d.	mean	s.d.
Fusitriton oregonensis (M)	0.00	0.00	0.00	0.00	3.00	2.37
Nucella lima (M)	0.83	1.33	0.83	0.98	1.67	2.25
Hermit crabs (M)	0.33	0.82	0.00	0.00	0.33	0.82
Leptaterias hexactis (M)	0.00	0.00	0.17	0.41	0.17	0.41
Katharina tunicata (M)	1.80	0.84	1.00	1.26	1.33	1.63
Limpets (M)	3.50	3.21	0.33	0.82	0.33	0.82
Evasterias troscheli (M)	0.00	0.00	0.00	0.00	0.17	0.41
Mopalia ciliata (M)	0.17	0.41	0.17	0.41	0.00	0.00
Gastropod snail (M)	0.17	0.41	0.00	0.00	0.00	0.00
Tonicella lineata (M)	0.33	0.52	0.00	0.00	0.00	0.00
Urticina crassicornis (S)	0.17	0.41	0.17	0.41	0.00	0.00
Balanus glandula (S)	101.67	74.88	31.17	34.41	38.67	31.60
Mytilus trossulus (S)	90.00	92.26	58.33	74.74	11.33	11.08
Metridium senile (S)	0.00	0.00	0.00	0.00	0.17	0.41
High						
	mean	s.d.	mean	s.d.	mean	s.d.
Katharina tunicata (M)	3.50	3.83	0.50	1.22	2.00	3.10
Limpets (M)	2.17	2.32	0.00	0.00	4.67	4.41
Onchidella borealis (M)	0.00	0.00	0.00	0.00	1.83	2.40
Siphonaria thersites (M)	0.00	0.00	0.00	0.00	0.33	0.52
Littorina sitkana (M)	0.00	0.00	0.00	0.00	0.17	0.41
Leptasterias hexactis (M)	0.33	0.52	0.00	0.00	0.00	0.00
Gatropod snail (M)	0.50	0.84	0.00	0.00	0.00	0.00
Mytilus trossulus (S)	90.00	96.26	0.17	0.41	8.33	20.41
Balanus glandula (S)	210.00	73.89	65.80	72.62	91.67	88.36



A.



B.

Figure 2: Density (#/unit area) present by mobility in the low (A) and high intertidal zones (B). Quadrats (0.30-m x 0.30-m) were haphazardly placed in each zone in Kasitsna Bay, near Chiton Rock (n=6). Error bars represent standard deviation.

DISCUSSION

Freezing tolerance has been studied in both terrestrial and submerged marine systems (5, 8, 9, 18, 20, 22, 26, 27, 34, 50, and many others). In these systems, freeze exposure is typically slow (taking weeks or months to occur) and/or prolonged (for weeks, months or even longer). Typically the organisms have time to acclimate to the freezing temperature and/or they will stay tolerant for prolonged periods of time. Rocky intertidal habitats are very different from these systems. The intertidal zone is one of the most physiologically challenging environments in which to live. During low tide, organisms can be subjected to very low air temperatures, during which there is a risk of freezing. While some invertebrates inhabiting this zone have clearly developed behavioral or physiological adaptations to survive, the effect of freezing temperatures on arctic and sub-arctic intertidal organisms is not well understood.

Varying degrees of freeze tolerance have been found in many intertidal invertebrate species. The barnacle *Semibalanus balanoides* has a mean lethal temperature of –18.6°C, and was found to be a freeze tolerant rather than freeze avoiding species (16). This lethal temperature is similar to what was found in the current study for *Mytilus trossulus* and *Balanus glandula*; they too are freeze tolerant, rather than freeze avoiding species. In another study (44) *S. balanoides* was not able to survive to –20°C, however in this study, *B. glandula*, a similar species survived to –20°C for up to six hours during winter. *Semibalanus balanoides* does not have a calcareous base plate whereas *B. glandula* does, so perhaps this base plate helps protect the tissue from freeze damage due to inoculative freezing or by keeping the organism at a higher temperature. With inoculative freezing an ice crystal forms in the environment, frozen seawater then touches the organism and results in seeding ice-crystals within the organism. Base plates also may keep organisms at a slightly higher temperature by separating them from the rock surface. In another study (done in Japan), 10 out of 48 intertidal species did not survive freezing to –10°C for one day without injury (47). These species included the barnacles *Chthamamlus dalli* and *Semibalanus carious*, the mussel *Mytilus grayanus*, the limpet *Collisella pelta*, the snails *Littorina squalida* and *Littorina atkana*, and the coelenterates *Anthopleura*

midori, *Anthopleura pacifica* and *Actina equina*. These species inhabit a similar tidal height to the organisms in the current study. In another study (on the east coast USA), *Mytilus edulis* was found to survive –10°C for up to 24 hours (51). The survival of *M. edulis* was similar to this study for the limpets, *M. trossulus, B. glandula* and to some extent *Protothaca staminea* (Tables 1 & 3). Limpet survival probably mirrored mussel survival because the majority of the limpets were collected off mussel shells, and in the same zone as *B. glandula*.

Thirty-four of the 48 species studied by Tanno and Wada (47) could not survive freezing to –5°C for one day. These species included the coelenterates *Tubularia venusta* and *Sertularella miurensis*; annelids *Nereis ezoensis*, *Potanilla myriops*, *Audoiuna conosa*, *Hydroides ezoenses* and *Physcosoma yezoense*; arthropods *Idotea japonica*, *Caprella* sp., *Orchestia platensis*, *Pagurus sammuellis*, *Telemessus cheiriagonus*, *Hemigrapsus pencillatus* and *Gaetice depressus*; molluscs *Acanthochiton rubrolineatus*, *Lepidozona albrechi*, *Pecten yessoensis*, *Clinocardium californiense*, *Entodesma maviculoides*, *Haliotis discus hannai*, *Cellana toreuma*, *Calorostoma argyrostoma lischkei*, *Omphalius rustica*, *Fusitriton oregonensis*, *Buccinum schantaricum* and *Aplysia kurodai*; echinoderms *Asterina pectinfera*, *Aphelasterias japonica*, *Strongylocentrous nudus*, *Strongylocentrotus intermedius*, *Stichopus japoniscs*, *Cucumaria japonica* and *Cucumaria chronhjelmi*; and the protochordate *Halocynthia hilgendorfi*. The lack of survival was probably because these organisms normally live below the low tide line or in tide pools, so they do not normally experience freezing temperatures. Living subtidally during the winter could explain why *F. oregonensis* had better survival in April/May 2003. Late spring/early summer is the time when *F. oregonensis* migrates from the subtidal into the intertidal for mating. Also in this study, the low survival of *Leptasterias hexactis* to freezing temperatures might be caused by habitat preference. *Leptasterias hexactis* was usually found under rocks, as opposed to exposed emergent surfaces, suggesting that microhabitats may be important in damping temperature extremes (30). The availability of microhabitats have been shown to help organisms thermoregulate in warmer climates, so this concept also might apply to colder climates.

Some of Tanno and Wado's (47) species live inhabit sand, which can act as a natural insulator, providing warmer conditions. In the current study, *Protothaca staminea* survival was higher than that of the other species tested (Table 1 & 3). The difference was probably because *P. staminea* utilized sand for insulation, which was found to be warmer than air. In this study, I rarely observed sand to freeze, though a sheet of ice usually formed on top of the sand with water remaining in the interstitial spaces. Subzero temperatures may cause this ice sheet to form rapidly and thus shield water and sand below it from the cold air. When the air was very cold, ice formed on top of the sand as the water receded. A slower freeze, with no ice insulation, resulted in the sand being frozen.

Four of the 48 species studied by Tanno and Wada (47) survived at -5°C, but not at -10°C. These species included the molluscs *Arca boucardi*, *Monodonta labio*, *Serpulorbis imbricatus* and *Anomia lischkei*. This pattern is similar to the mixed survival results seen with *Katharina tunicata* and *Fusitriton oregonensis* in the current study. These species can regulate where they live in the intertidal to some extent, and can utilize microhabitats to help them survive freezing, which could cause individual variation in survival as observed in this study.

In nature, freezing is not limited to a single night or tide. In Alaska, there are two low tides per day that may subject intertidal organisms to freezing temperatures. Unlike terrestrial and submerged marine species, intertidal organisms undergo multiple freeze events, usually with each low tide in the winter. *Semibalanus balanoides*, *Littorina littorea* and *Littorina rudis* can survive freezing to -10°C continuously for several days (44). Although the current study did not utilize prolonged freezing periods because the test animals do not experience them in nature, the repeated freezing experiments of *M*ytilus *trossulus* and *Balanus glandula* (Table 2) suggest that they can survive freezing more than once to -10°C. In a pilot study, *M. trossulus* was able to survive to -20°C for two hours during other times of the year besides December (pers. obs.). This implies that the length of time frozen at a certain temperature is important. Without protection, six hours may be fatal because a critical amount of free water is frozen. During most low tides in this region, *M. trossulus* would be emergent for an average of six hours.

The ability to survive freezing, or multiple freeze events, has been shown to change seasonally for many intertidal species. In this study, differential seasonal freeze tolerance was demonstrated by *Mytilus trossulus* and *Balanus glandula* with higher survival in winter compared to summer. Other intertidal species that have shown seasonal changes in freeze tolerance are *Littorina squalida* and *Collisella pelta*, two molluscs that also have more tolerance to freezing in winter than in summer (47). Somme (44) found freezing survival to be greatest in the middle of winter, when *Balanus balanoides*, *Littorina littorea*, and *Littorina rudis* survived freezing to -10°C for several days. *Littorina littorea* was shown to have seasonal differences in freeze tolerance by Murphy (41), with survivability greatest in the winter months. *Littorina littorea* collected between September and March had an LD_{50} temperature of ~-13°C, while those collected between May and July could not survive freezing below -11°C (41). A difference in seasonal survival is usually due to the animal acquiring or creating compounds that aid them in freeze survival (6, 13, 18, 36, 39).

Many researchers have investigated the physiological basis for freeze tolerance. Salinity acclimation (39), the binding of calcium ions (40), and anaerobic end products (lactate, 36) have all been reported as aids for surviving freezing. Increasing salinity is a mechanism to increase freeze tolerance (up to a lethal limit) (39). Binding calcium ions to membranes can impart extra protection against freeze damage (40). Anaerobic end products appear to act in a manner similar to that of sugars such as glycerol. Freeze tolerance has also been shown to be a residual trait in some organisms. For example, the limpet *Collisella digitalis* from the California coast was found to have a supercooling point as low as -12.3°C (42). These organisms rarely experience freezing temperatures where they were collected, so this low supercooling point may provide evidence that *C. digitalis* is a northern species that has extended its range southward. Hayes and Loomis (29) found that *Melampus bidentatus* acquired freeze tolerance only after exposure to 0°C at which time an ice nucleator appeared. The lowest lethal temperature of snails held at 0°C was not significantly different than the lethal temperature of winter-collected snails. In addition, exposure to 0°C lowered the supercooling point of summer collected snails

(29). The current study found that exposure to air did not influence the acquisition of freeze tolerance in *Mytilus trossulus* (Table 4). This is illustrated by similar freezing survival rates in individual *M. trossulus* collected from the dock, a continuously submerged habitat, and individuals who were exposed to low air temperatures.

Cyro-protection (having compounds that prevent freezing) is an important survival mechanism at low air temperatures. An interesting form of protection was discovered in the Antarctic limpet, *Patinigera polaris* (28), which secretes a water-impermeable mucus that contains a glycoprotein thermal hysterisis factor allowing it to resist freezing to -10°C. A significant increase has been shown in survivorship of limpets that had the mucus compared to those that had it removed (28). In the current study, cryo-protection may be present in *Mytilus trossulus* as its supercooling point was significantly lower than that of seawater and there was also a small thermal hysteresis (difference between melting and freezing points) factor present. Aunaas (2 & 3) and Hayes & Loomis (29) felt that differences in seasonal supercooling points are due to the presence of an ice nucleator protein (a protein that causes nucleation at a higher temperature than is typical), which was found only in winter. In the current study, survival did not vary by habitat or supercooling point, further supporting the hypothesis that exposure to low air temperature is not stimulating the freeze tolerance in *M. trossulus*.

Another method employed by intertidal organisms to avoid freezing is migration out of the intertidal (41). In the current study it appears that some intertidal species could be moving to the low intertidal or shallow subtidal during the winter to avoid freezing temperatures. *Fusitriton oregonensis*, *Nucella lima*, *Onchidella borealis*, *Siphonaria thersites* and *Littorina sitkana* averaged a higher density in either the high or low zones during April/May 2003 when compared to January 2003. More data are needed to confirm this seasonal movement in populations, especially during summer months to determine if there are significant differences in density between the zones or by mobility.

The repeated-freezing experiment combined with the ability of some species to survive freezing to -20°C implies that a very cold freeze (approximately -20°C or lower) is needed to cause extensive winterkill in the intertidal in this region. During

the period of the current study, the air temperature in the study area never exceeded -12°C during a low tide series (Patterson, unpublished data). However, winterkill has been reported in the study area, particularly during the winter of 1989 (11), the same year as the Exxon Valdez oil spill. The temperatures during this month reached -31°C (weather.com). During this oil spill, it was difficult for researchers to differentiate between freeze effects and oil effects because so little is known about freeze tolerance in most intertidal organisms (Highsmith pers. comm.). A similar situation with a concurrent oil spill and freeze event occurred in Washington State (19). These oil spills demonstrate the need for prespill freeze data. The current study provides some insight into freezing tolerance in the Alaskan intertidal, demonstrating that some intertidal invertebrates can survive subzero temperatures but there is a lower lethal limit. It can also be speculated that any additional stress (such as oil) would only increase mortality at the lower temperatures, as observed by the mixed survival seen at these temperatures.

In summary, this study demonstrated that freeze tolerance exists in the Alaskan intertidal and that some species exhibit seasonal changes in freeze tolerance. The sessile organisms *Mytilus trossulus* and *Balanus glandula* can survive repeated freezing to -10°C in a laboratory setting. Exposure to cold air temperatures did not change freeze tolerance in *M. trossulus*. Also in a laboratory setting, the supercooling points of *M. trossulus* and *P. staminea* were not significantly different between January and March, but were higher than seawater. Interestingly, exposure to low air temperatures (0, -10, -20°C) also did not affect the supercooling point in *M. trossulus*. Migration of some mobile organisms may cause a difference in species distribution in the intertidal as more mobile organisms were present in April/May 2003 than in the proceeding January. Implications of the laboratory experiments were demonstrated in the field observations. Mobile organisms, which typically did not show freeze tolerance in the laboratory experiments, were found to have different distributions between the summer and winter, while sessile organisms had consistent spatial distributions regardless of season.

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